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Phosphatidylcholine-specific phospholipase C, p53 and ROS in the association of apoptosis and senescence in vascular endothelial cells

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Abstract **Previously, we found that phosphatidylcholine-specific phospholipase C (PC-PLC) participated in apoptosis signaling of vascular endothelial cells (VECs). Here, to explore whether PC-PLC is involved in the association of apoptosis and senescence in VECs, we analyzed p53 expression and intracellular reactive oxygen species (ROS) levels in young and senescent VECs before and after inhibiting PC-PLC activity. The results showed that suppressing PC-PLC inhibited apoptosis and the elevation of p53 expression induced by apoptosis in young cells, but not in senescent cells, and that inhibiting PC-PLC depressed intracellular ROS levels both in young and senescent cells. The data suggested that PC-PLC was involved in the association of apoptosis and senescence. Its function might be closely related to the level of p53 in VECs.**

Keywords: Phosphatidylcholine-specific phospholipase C; Senescence; Apoptosis; Vascular endothelial cells; p53; ROS

1. Introduction

Replicative senescence of human cells in primary culture is a widely accepted model for studying the molecular mechanisms of human aging. Many results showed that there are profound cell type-specific differences in the senescence program [1]. Therefore, it is necessary to select an appropriate model for understanding the senescence mechanism of certain histotypes.

It has been reported that apoptosis plays a key role for aging in vivo. There are many reports respectively directed to signaling pathways of apoptosis and senescence. The relationship between the two processes, however, is not well known. Unlike fibroblasts, vascular endothelial cells undergo aging associated with apoptosis in vitro. It provides a good model system to study the relationship between senescence and apoptosis in vascular aging at both cellular and molecular levels [2]. In this study, to find the key factors that associate senescence and apoptosis, we selected VECs as the most appropriate model.

PC-PLC, an important member of phospholipase C family, might be involved in age-related signal transduction in human lung fibroblasts and rat hepatocytes [3]. But it is not known whether and how PC-PLC changes in the senescence of VECs.

We have been studying the function of PC-PLC in apoptosis signaling of VECs. The results showed that PC-PLC was a key factor in apoptosis signal transduction pathways [4–10]. In this study, we focused on exploring whether PC-PLC is involved in the association of apoptosis and senescence in VECs.

In previous studies, we found that p53 played a key role in the regulation of VEC apoptosis [7–9,11,12]. To understand the mechanism by which PC-PLC regulates senescence and apoptosis in VECs, we investigated the relationship between PC-PLC and p53 during VEC aging.

Free radical theory of aging pointed out that reactive oxygen species (ROS) were major factors responsible for human aging. Moreover, ROS were implicated as potential modulators of apoptosis [13]. To understand the relationship between PC-PLC and ROS in the association of apoptosis and senescence of VECs, we examined the changes of ROS levels in young and senescent cells before and after the inhibition of PC-PLC activity.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained as described previously [14]. The cells were cultured in MCDB131 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 70 ng ml^{-1} FGF and 100 μg ml^{-1}heparin.

2.2. PC-PLC activity assay

PC-PLC activity was detected as described by Wu et al. [15].

2.3. Apoptosis induction and inhibition

Rattlesnake venom can specifically trigger apoptosis in HUVECs [16]. D609, a specific inhibitor of PC-PLC, could suppress young VEC apoptosis induced by deprivation of FGF and serum [5,6]. In this study, we selected this model to study the relationship between senescence and apoptosis in HUVECs. When the cells reached sub-confluence, they were treated by the following three ways: (a) As a control group, cells were cultured in basal MCDB131 medium (without FGF and serum). (b) Cells were treated with rattlesnake venom of 2 μg ml^{-1} for 6 h. (c) Cells were incubated with rattlesnake venom of 2 μg ml^{-1} and D609 of 10 μg ml^{-1} for 6 h.

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Abbreviations: HUVEC, human umbilical vein endothelial cell; PDL, population doubling level; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species; SA-β-Gal, senescence-associated β-galactosidase; VEC, vascular endothelial cell

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2.4. Analysis of nuclear fragmentation

The cells treated, in the three ways mentioned above (in Section 2.3), for 6 h were stained with acridinorange for 5 min, and observed under laser scanning confocal microscope (Zeiss, LSM510).

2.5. ROS assay

A fluorescent probe, 2′,7′-dichlorodihydrofluorescein (DCHF), which could be oxidized into 2′,7′-dichlorofluorescein (DCF) by the intracellular ROS while entering into the cell, was used for the assessment of intracellular ROS formation in cultured VECs. ROS assay was performed as described previously [17]. The levels of ROS in VECs were quantified using the software of Zeiss LSM510.

2.6. Western blot analysis

The Western blot analysis was performed as described previously [18]. The relative amount of proteins was analyzed by using Imagetool software.

2.7. Statistics analysis

The results were expressed as means ± S.E. Statistical analysis was performed by *t*-test, and differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of VEC senescence

VEC senescence is identified by the senescence-associated β-galactosidase (SA-β-Gal) as recognized in the art [19]. In this study, the activity of SA-β-Gal was examined in PDL 10, PDL 20 and PDL 36 cells respectively. As shown in Fig. 1A–C, SA-β-Gal activity was remarkably increased concomitant with the morphological changes of senescent cells. Based on these results, in the following experiments, we selected PDL 16 cells and PDL 36 cells as young and senescent representatives respectively.

3.2. The activity of PC-PLC remarkably decreased with VEC aging

To know whether and how PC-PLC changes with VEC senescence, we examined the activity of PC-PLC in PDL 16 and PDL 36 HUVECs respectively. The results showed that PC-PLC activity in PDL 36 HUVECs was much lower than that in PDL 16 HUVECs (Fig. 1D).

3.3. Suppressing PC-PLC inhibited apoptosis in PDL 16 cells but not in PDL 36 cells

Apoptotic body formation and the nuclear fragmentation are the typical characteristics of VEC apoptosis [6,8,9]. In this study, these morphological changes in VECs treated with rattlesnake venom were observed. The results showed that the apoptosis both in PDL 16 and PDL 36 VECs could be triggered by rattlesnake venom of 2 μg ml^{-1} (Fig. 2A(b) and (e); Fig. 2B(b) and (e)). In PDL 16 VECs, after the cells were exposed to D609 of 10 μg ml^{-1}for 6 h, the apoptosis was obviously suppressed and fewer apoptotic bodies were observed. In PDL 36 VECs, however, the apoptosis was not suppressed by D609 of 10 μg ml^{-1} (Fig. 2A(c) and (f); Fig. 2B(c) and (f)).

3.4. Suppressing PC-PLC inhibited p53 expression induced by apoptosis in PDL 16 cells but not in PDL 36 cells

To understand why inhibition of PC-PLC can not suppress the apoptosis in PDL 36 cells, we examined the expressions of p53 protein in PDL 16 and PDL 36 cells treated in the three

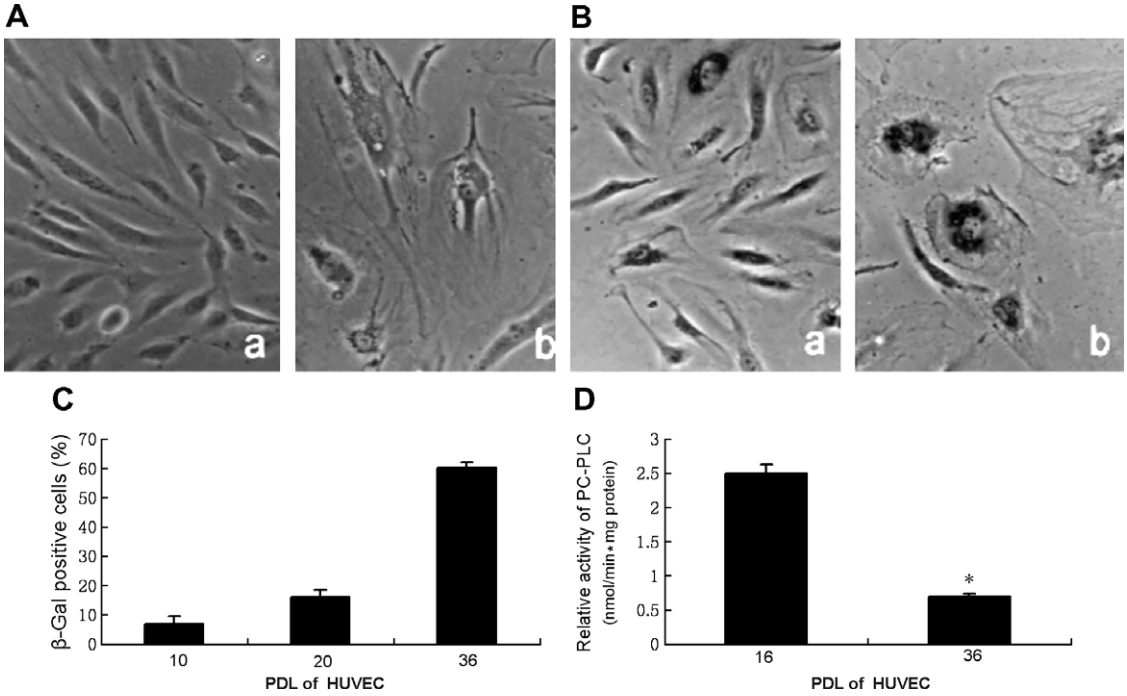

Fig. 1. (A) The morphological changes during VEC aging. PDL 36 cells (b) were bigger and more spread than PDL 16 cells (a) in morphology. (B) SA-β-Gal activity analysis. There were more β-Gal positive cells among PDL 36 cells (b) than those among PDL 16 cells (a). (C) The percentage changes of β-Gal positive cells with cell aging. The percentage of β-Gal positive cells among PDL 10, 20 and 36 cells amounted to 8%, 16% and 65% respectively, suggesting that the cell gradually became senescence. (D) The activity changes of PC-PLC during VEC aging. PC-PLC activity in PDL 16 cells was 2.5 nmol/min mg protein, whereas, it was 0.7 nmol/min mg protein in PDL 36 cells. The activity of PC-PLC decreased remarkably with VEC aging.*$P < 0.01$ ($n = 3$).

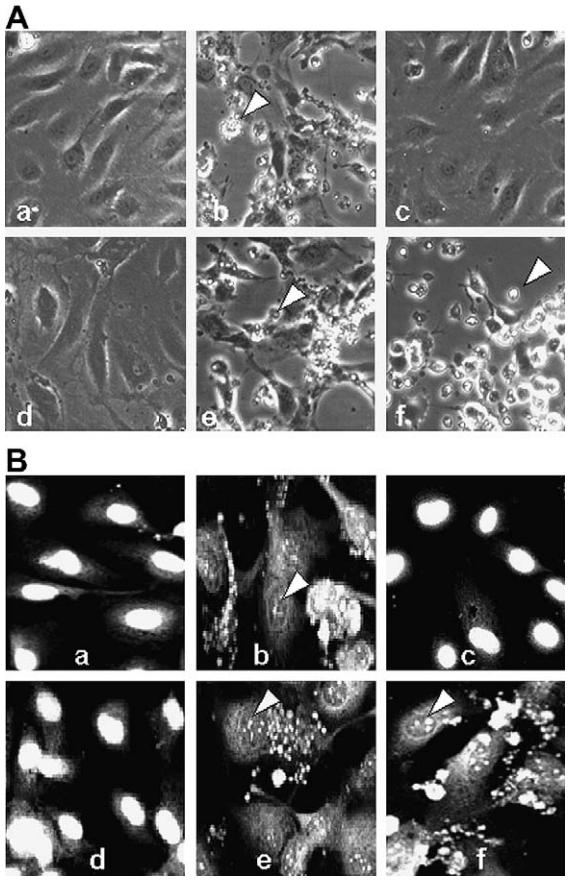

Fig. 2. (A) The morphological changes associated with apoptosis. (a) PDL 16 cells cultured in the basal medium for 6 h; (b) Apoptosis was induced by rattlesnake venom of 2 μg ml^{-1} in PDL 16 cells; (c) The apoptosis was inhibited by D609 of 10 μg ml^{-1} in PDL 16 cells. (d) PDL 36 cells cultured in the basal medium for 6 h; (e) Apoptosis was also induced by rattlesnake venom of 2 μg ml^{-1} in PDL 36 cells; (f) The apoptosis was not inhibited by D609 of 10 μg ml^{-1} in PDL 36 cells. The detachment of cells and apoptotic bodies were observed (b, e, f). The arrowheads indicate the apoptotic bodies. (B) The nuclear changes associated with apoptosis. (a) The nuclei of PDL 16 cells cultured in the basal medium for 6 h. About 90% nuclei of the cells remained intact. (b) The nuclear fragmentation associated with apoptosis induced by rattlesnake venom of 2 μg ml^{-1} was noted in PDL 16 cells. (c) The nuclear fragmentation was inhibited by D609 of 10 μg ml^{-1} in PDL 16 cells. (d) The nuclei of PDL 36 cells cultured in the basal medium for 6 h; (e) The nuclear fragmentation was induced by rattlesnake venom of 2 μg ml^{-1} in PDL 36 cells; (f) The nuclear fragmentation was not inhibited by D609 of 10 μg ml^{-1} in PDL 36 cells. The nuclear fragmentation and apoptotic bodies were observed (b, e, f). The arrowheads show the nuclear fragmentations.

ways mentioned above (in Section 2.3). The results showed that the expressions of p53 were obviously increased during apoptosis both in young and senescent cells. The elevation was suppressed by inhibiting PC-PLC in PDL 16 cells, but not in PDL 36 cells (Fig. 3).

3.5. D609 depressed the increase of ROS levels induced by apoptosis both in young and senescent cells

ROS are very important signal molecules both in apoptosis and senescence. To know whether ROS are involved in the association of apoptosis and senescence mediated by PC-PLC, we examined the levels of intracellular ROS in PDL 16 and PDL 36 cells treated in the three ways mentioned above

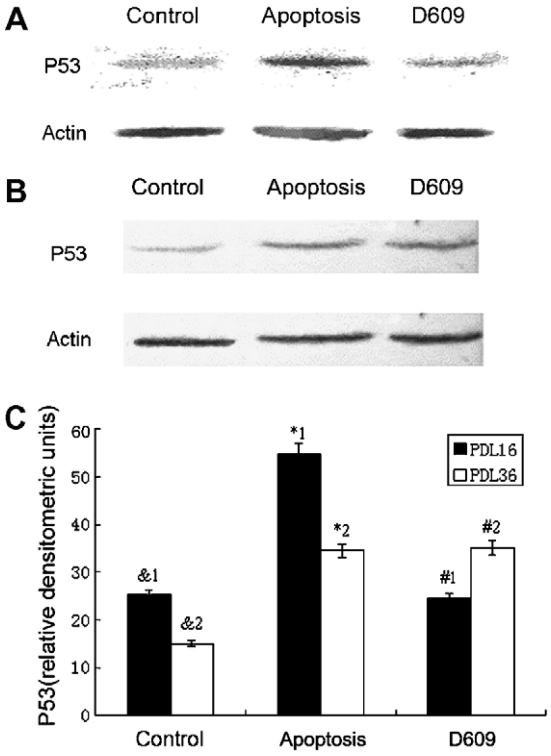

Fig. 3. Suppressing PC-PLC could inhibit p53 expression induced by apoptosis in PDL 16 cells but not in PDL 36 cells. (A) p53 protein from PDL 16 VECs; (B) p53 protein from PDL 36 VECs. (A) and (B), Western blot photos. (C) The quantity of p53 protein. Control, p53 protein from the cells cultured in the basal medium. Apoptosis and D609, p53 protein from the apoptotic cells and D609-treated cells respectively. *1, $P < 0.01$ vs &1; #1, $P > 0.05$ vs &1. *2, $P < 0.01$ vs &2; #2, $P < 0.01$ vs &2; #2, $P > 0.05$ vs *2 ($n = 3$).

(in Section 2.3). As shown in Fig. 4, the levels of ROS were clearly elevated during apoptosis both in young and senescent cells. The elevation was depressed by inhibiting PC-PLC both in PDL 16 and PDL 36 cells. But the level of ROS in PDL 36 cells treated with D609 was more dramatically depressed than that in PDL 16 cells treated with D609.

4. Discussion

It is well known that there are clearly profound cell type-specific differences in the senescence program [1]. In this study, to study the relationship between senescence and apoptosis in vascular aging, we selected HUVEC as the experimental model.

Our recent results showed that PC-PLC had important roles in HUVEC apoptosis [4–10]. But, so far, it is not known whether and how PC-PLC participates in the senescence of VECs, especially the role of PC-PLC in the association of apoptosis and senescence is completely unknown. In this study, we found that PC-PLC activity decreased remarkably in senescent VECs and that suppressing PC-PLC could inhibit apoptosis in the young cells, but could not in the senescent cells. The data suggested that PC-PLC was a key factor in the association of apoptosis and senescence. This finding provides the new evidence for understanding the relationship between apoptosis and senescence in VECs.

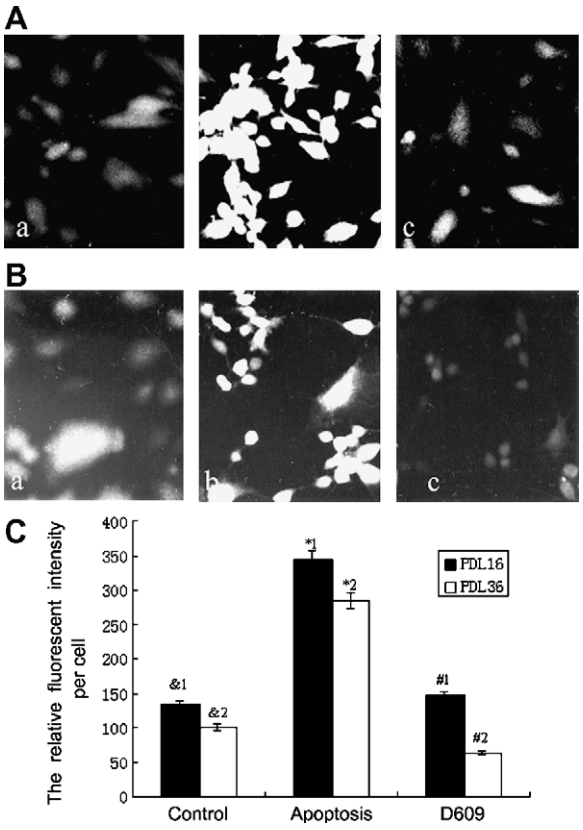

Fig. 4. D609 depressed the increase of ROS levels induced by apoptosis both in young and senescent cells. Fluorescent micrographs show the relative intensity of ROS in PDL 16 cells (A) and in PDL 36 cells (B). (a) Cells cultured in the basal medium for 6 h, (b) and (c) cells treated with rattlesnake venom of 2 $\mu g \cdot ml^{-1}$ in the absence or presence of D609 of 10 $\mu g \cdot ml^{-1}$ respectively. (C) ROS levels in PDL 16 and PDL 36 cells. Control, the levels of ROS in the cells cultured in the basal medium; Apoptosis and D609, the levels of ROS in the cells treated with rattlesnake venom of 2 $\mu g\ ml^{-1}$ in the absence or presence of D609 of 10 $\mu g\ ml^{-1}$ respectively. *1, $P < 0.01$ vs &1; #1, $P < 0.01$ vs *1; #1, $P > 0.05$ vs &1. *2, $P < 0.01$ vs &2; #2, $P < 0.01$ vs *2; #2, $P < 0.05$ vs &2 ($n = 3$).

Our previous studies showed that p53 played a key role in regulating apoptosis induced both by deprivation of survival factors and by rattlesnake venom in VECs, and that inhibiting p53 expression by suppressing PC-PLC with D609 could block apoptosis in young VECs [7–9,12]. In this study, the result showed that the expression of p53 was markedly elevated during apoptosis in PDL 16 cells, and that the increase could be suppressed by inhibiting PC-PLC. This result is consistent with our previous report. Here, it is our new finding that the expression of p53 was also remarkably elevated during apoptosis in senescent cells, but the elevation could not be suppressed by inhibiting PC-PLC.

Several experimental results showed that, besides as inhibitor of PC-PLC, D609 is also an antioxidant [20]. Thus, in addition to its inhibitory effect on PC-PLC, D609 may also prevent apoptosis as an antioxidant. It has been reported that elevation of intracellular ROS can trigger apoptosis in many types of cells, including VECs [17,18,21]. Consistent with these data, our results showed that the levels of ROS were markedly increased during apoptosis. It is well known that the balance of intracellular ROS is important to cell survival. In this study, the levels of ROS in the cells treated with D609 could be recovered to the levels of control cells in PDL 16 cells. But in PDL 36 cells, the levels of ROS in the cells treated with D609 were more depressed than those in the control cells. When the intracellular ROS levels were too low, VEC apoptosis also took place [10]. This is consistent with our previous report.

It is reported that apoptosis and subsequent loss of irreplaceable cells may be an important mechanism of aging in mammals [22]. There are two major pathways for initiation of apoptotic signaling. One is the cell death receptor mediated apoptotic signaling and the other is the mitochondrial cell death pathway. Ceramide, ROS, and p53 are related to the mitochondrial cell death pathway. Ceramide, a second messenger-like molecule, is generated by sphingomyelin (SM) breakdown catalyzed by sphingomyelinase (SMase) [23]. SMase activation is secondary to the generation of 1,2-diacylglycerol(DAG)produced by a TNF-responsive PC-PLC. Besides triggering rapid induction of nuclear NF-κ B activity, ceramide can also cause some change in mitochondria via disruption of oxidative-phosphorylation and electron transport leading to apoptosis. Thus, PC-PLC was closely related to mitochondrial cell death pathway. It has been reported that ROS levels are important in moderating the rate of Fas-dependent apoptosis and may play a pivotal role in cell survival [24]. Our previous study suggested that PC-PLC was an important element in VEC apoptosis mediated by Fas [10]. Therefore, the function of PC-PLC may associate with ROS through mitochondria during apoptosis. It is well known that ROS can cause damage to DNA. P53 is often expressed in cells with DNA damage [25]. Cells undergo p53-dependent apoptosis through oxidative damage, such as ROS. P53 plays essential and central roles in the signaling pathways of apoptosis and senescence. The increase of p53 expression ultimately induces replicative senescence in many kinds of cells. Thus, PC-PLC possibly mediated the induction of apoptosis by cooperation with ROS and p53.

In this study, we found that the activity of PC-PLC in aged VECs was much lower than that in young VECs. The data suggested that the PC-PLC in low activity possibly could not regulate the ROS levels through mitochondria and then the interaction between p53 and PC-PLC could not take place. This finding indicated that young cells and senescent cells are going to different apoptotic signaling pathways. We will determine these possible distinct signaling pathways in our future studies.

Taken together, our findings provided the new evidence for understanding the relationship between apoptosis and senescence, and encourage us to elucidate the interaction among PC-PLC, p53 and ROS in the association of apoptosis and senescence.

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